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Impact of *Pseudomonas fluorescens* strain CHA0 and a derivative with improved biocontrol activity on the culturable resident bacterial community on cucumber roots

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Abstract

Information on the effects of released wild-type or genetically engineered bacteria on resident bacterial communities is important to assess the potential risks associated with the introduction of these organisms into agroecosystems. The rifampicin-resistant biocontrol strain *Pseudomonas fluorescens* CHA0-Rif and its derivative CHA0-Rif/pME3424, which has improved biocontrol activity and enhanced production of the antibiotics 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt), were introduced into soil microcosms and the culturable bacterial community developing on cucumber roots was investigated 10 and 52 days later. The introduction of either of the two strains led to a transiently enhanced metabolic activity of the bacterial community on glucose dimers and polymers as measured with BIOLOG GN plates, but natural succession between the two sampling dates changed the metabolic activity of the bacterial community more than did the inoculants. The introduced strains did not significantly affect the abundance of dominant genotypic groups of culturable bacteria discriminated by restriction analysis of amplified 16S rDNA of 2500 individual isolates. About 30–50% of the resident bacteria were very sensitive to Phl and Plt, but neither the wild-type nor CHA0-Rif/pME3424 changed the proportion of sensitive and resistant bacteria in situ. In microcosms with a synthetic bacterial community, both biocontrol strains reduced the population of a strain of *Pseudomonas* but did not affect the abundance of four other bacterial strains including two highly antibiotic-sensitive isolates. We conclude that detectable perturbations in the metabolic activity of the resident bacterial community caused by the biocontrol strain CHA0-Rif are (i) transient, (ii) similar for the genetically improved derivative CHA0-Rif/pME3424 and (iii) less pronounced than changes in the community structure during plant growth.

Keywords: *Pseudomonas*; Biocontrol; Improved biocontrol activity; Effect on resident bacteria; Diversity; Restriction analysis of amplified ribosomal DNA

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1. Introduction

The commercial application of beneficial bacteria for biological control or bioremediation requires the release of large numbers of cells of wild-type or genetically engineered strains into the soil ecosystem. The ecological safety of such releases should be assessed and studies on microbial inoculants have therefore become a novel topic in ecotoxicology. Introduced bacteria might outcompete a certain resident subpopulation for nutrients and for space. Further, toxic compounds (e.g. antibiotics or degradation products of xenobiotics) produced by the introduced strains could affect sensitive microorganisms. Most studies on the effects of genetically engineered microorganisms (GEMs) on the resident bacterial community published have focused on genetically engineered strains which had only a marker gene inserted and no or little effect of these GEMs on the resident microbial community has been reported [1–5]. In certain cases, strains were investigated which were engineered to metabolize specific substrates present in the soil and significant ecological effects of these inoculants were observed [6–8].

Biological control of soil-borne plant diseases with strains of *Pseudomonas* has been studied intensively in recent years [9,10]. Many of the most promising biocontrol strains produce antimicrobial metabolites that are relevant for their biocontrol activity [9,11] and are toxic to a range of different soil microorganisms in vitro [12,13]. The establishment of biocontrol bacteria in the rhizosphere might therefore not affect exclusively the target pathogen but also non-target microorganisms. On the other hand, extractable amounts of antibiotics on roots are generally low [12,14–16], and their activity might be restricted to specific microsites on the roots [10,17]. A number of *Pseudomonas* strains have been genetically engineered to produce additional antibiotics or increased amounts of these metabolites resulting in a considerable improvement of their biocontrol performance [18–24]. Data on non-target effects of these modified strains on the diversity of resident microbial populations in soil, in particular on antibiotic-sensitive groups, have not been reported.

Both culture-based methods and community approaches without in vitro cultivation can be used to study the diversity of microbial communities [25,26]. In contrast to the soil environment, where a significant fraction of the bacteria is unculturable, results obtained with direct counts and plate counts are quite similar for the bacterial community of the rhizosphere [27,28] and methods which describe individual isolates are routinely used to study bacterial communities of this ecological niche [29–32]. Both phenotypic tests (e.g. carbon source utilization, antibiotic resistance or fatty acid methyl ester profiles [29,31,32]) and genetic fingerprinting techniques such as restriction analysis of amplified ribosomal DNA (ARDRA) [30,33] can be used. Other methods describe the overall function or composition of a community. A community approach which has become commonly used measures the metabolic properties of microbial communities by inoculation of BIOLOG microplates with a complex community [1,2,34,35].

Here we report on the impact of *Pseudomonas fluorescens* CHA0 and its derivative CHA0/pME3424 on the resident bacterial community on cucumber roots using a range of these tests. Strain CHA0 is an effective biocontrol agent, which protects plants from various root diseases [10,11]. The functionally improved strain CHA0/pME3424 contains an extra copy of the homologous *rpoD* gene, encoding the housekeeping sigma factor σ^{70}, inserted into the oligo-copy vector pVK100. Its disease suppressive capacity is 30–60% improved ([15,23,36]; our unpublished results) and its production of the antibiotics 2,4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt) is severalfold increased. In previous studies, the ecological behavior and fate (e.g. vertical transport, survival, physiological activity, culturability and transfer of chromosomal genes) of strain CHA0 has been monitored in detail under growth chamber and field conditions [37–41]. The effects of CHA0 and CHA0/pME3424 on taxonomically closely related resident bacteria (i.e. pseudomonads) have been studied in microcosms [42]. The main purposes of this study were (i) to investigate the possible impact of *P. fluorescens* CHA0 and its derivative on the metabolic profile and on the abundance of dominant genotypic groups of culturable resident bacteria colonizing cucumber roots and (ii) to evaluate whether these strains affect resident bacteria that are sensitive to the antibiotics produced by the inoculants. Experiments involved microcosms with both a natural and a synthetic bacterial community.

2. Materials and methods

2.1. Organisms and culture conditions

A previously described derivative of *P. fluorescens* strain CHA0 selected for spontaneous resistance to rifampicin (CHA0-Rif) [37,38] was used for all experiments in this study. The plasmid pME3424 containing an extra copy of the *rpoD* gene [23] was introduced into CHA0-Rif and antibiotic overproduction was verified as described by Schnider et al. [23]. Both strains were routinely grown in nutrient yeast broth (NYB) [43] and on King's medium B agar (KMB) [44] containing 100 μg rifampicin per ml. For strain CHA0-Rif/pME3424 125 μg ml^{-1} tetracycline hydrochloride was added to the medium. For cultivation of all isolates from cucumber roots, 1/10 strength tryptic soy broth (Difco, Detroit, MI, USA; further referred to as TSB) and 1/10 strength tryptic soy agar solidified with 12 g extra agar per liter (further referred to as TSA) were used throughout this study.

Cucumber seeds (*Cucumis sativus* L. cv. Chinesische Schlange, Altdorfer Samen, Zürich, Switzerland) were surface-disinfested in 5% (w/v) sodium hypochlorite for 30 min, rinsed with sterile-filtered water and then germinated for 2 days on 0.85% water agar (Difco) at 24°C.

2.2. Microcosms with the natural bacterial community

Soil from Eschikon (Switzerland) used in this study was collected from the upper 20 cm of the soil profile, passed through a 5 mm mesh screen, and stored at 15°C prior to use. Soil properties are described elsewhere [37]. For soil inoculation, CHA0-Rif and CHA0-Rif/pME3424 were grown on KMB overnight at 27°C. Bacteria were removed from the plates, washed in sterile distilled water and the bacterial concentration was adjusted to 5×10^8 CFU ml^{-1}. Soil was sprayed with the bacterial suspension at a rate of 20 ml kg^{-1} to obtain a final concentration of 10^7 CFU per g soil. An equivalent volume of sterile distilled water was added to soil for the control treatment. Soil was thoroughly mixed during application. Quartz sand (50 ml) was added to clay pots (400 ml internal volume) and overlaid with 350 ml of soil. Two pre-germinated cucumber seedlings were planted per pot, and the pots were incubated in a growth chamber with 70% relative humidity, 16 h light (160 microeinstein m^{-2} s^{-1}) at 22°C, followed by an 8-h dark period at 18°C. Twice a week pots were irrigated from below with sterile distilled water. Plants were harvested after 10 and 52 days, and the bacterial populations on the roots were examined as described below. A total of 24 plants was analyzed per treatment, repetition and sampling time.

2.3. Enumeration and isolation of root-associated bacteria

The roots were washed gently for 10 s with sterile water to remove loose soil and then homogenized with a mortar and pestle for 30 s. The homogenate was diluted 100-fold in sterile distilled water and shaken at 300 rpm for 1 h on a rotary shaker. Ten-fold serial dilutions were spread-plated on TSA to isolate total culturable bacteria and on KMB containing 100 μg of rifampicin and 187.5 μg of actidione per ml to enumerate culturable cells of introduced CHA0-Rif or CHA0-Rif/pME3424. Plates were incubated at 24°C, and colonies were counted after 3 days. The rifampicin-resistant background populations in Eschikon soil were below the detection limit of 10^2 CFU g^{-1}. To evaluate plasmid loss of CHA0-Rif/pME3424, rifampicin-resistant isolates (12 isolates per root system at the first sampling date and a total of 200 isolates at the second sampling date) were transferred to KMB containing 125 μg ml^{-1} tetracycline hydrochloride.

In order to evaluate the abundance of dominant genotypes of resident bacteria, isolated bacterial colonies were picked randomly from final dilutions on TSA (12 colonies for each root system) and transferred to TSA master plates in a 6×8 pattern suitable for replica plating. Rifampicin resistance of the isolates was tested by replica plating on KMB amended with rifampicin, and resistant isolates were omitted in further evaluation. After 2 days of growth on the TSA master plates, the isolates were subcultured in microtiter dishes containing 100 μl of TSB per well. Isolates were then stored at −80°C after adding 50 μl of a sterile 60% glycerol solution to each culture.

2.4. Characterization of bacterial isolates

To determine the abundance of dominant genotypes of resident bacteria, the 3300 isolates obtained at the sampling date after 10 days were analyzed with the ARDRA technique as described in detail previously [45]. Briefly, bacterial cultures were grown for 48 h at 24°C in microtiter plates containing 100 μl of TSB per well and 0.5 μl of the cultures was transferred directly to PCR tubes for heat lysis in a total volume of 5 μl of lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Tween 20). The final volume of the PCR reactions was 20 μl. Primers with the following sequences were used to amplify the full length of the 16S rDNA gene: 5′-GCTCAGATTGAACGCTGGCG (forward) and 5′-CGGTTACCTTGTTACGACTTCACC (reverse) [45]. Restriction of 10 μl of the amplified product was performed in a total volume of 20 μl of restriction buffer with 2 U of either *Cfo*I or *Taq*I (Boehringer, Mannheim, Germany). Restriction fragments were separated on 2.5% agarose gels.

In order to determine the resistance of the same isolates to the antibiotics Phl and Plt produced by CHA0-Rif, 0.5 μl of a 2-day-old TSB culture of the strains was transferred to microtiter plates containing 100 μl of TSB with the appropriate amount of the respective antibiotic per well. Phl and Plt were synthesized and kindly provided by E. Burger, University of Geneva, Switzerland. Plates were shaken at 100 rpm for 3 days at 24°C and growth was assessed by measuring the optical density at 570 nm with an ELISA plate reader. Values below 0.05 were considered to show inhibition of growth of the respective isolates. Characterization of the individual bacterial isolates with ARDRA and evaluation of antibiotic resistance was performed only for the isolates obtained at the first sampling date.

2.5. Detailed characterization of selected isolates representing dominant ARDRA groups

From each of the 11 dominant ARDRA groups distinguished with *Cfo*I, three independent isolates were chosen. For each isolate, the inhibitory concentration of the antibiotics Phl and Plt was tested as described above. In addition, the in vitro inhibition of these isolates by CHA0-Rif and CHA0-Rif/pME3424 was assessed. Therefore, 5 μl of a washed culture of either CHA0-Rif or CHA0-Rif/pME3424 containing 5×10^8 CFU ml^{-1} was inoculated 10 mm from the edge of TSA plates and incubated for 24 h at 27°C. The colonies that grew (about 6 mm in diameter) were excised with a sterilized cork borer (10 mm internal diameter). The plates were then overlaid with TSA soft agar (0.6% agar) mixed 1:50 with an overnight TSB culture of the selected isolates. The radius of the inhibition zone from the edge of the excised zone was measured after 24 h of incubation at 27°C. Since the colonies were excised, only inhibition zones larger than 2 mm could be measured. For genus and species identification of the isolates the BIOLOG identification system was used as recommended by the manufacturer (BIOLOG Inc., Hayward, CA) with the exception that plates were incubated for 72 h, since it has recently been shown that for many environmental isolates stable positive or negative reactions are only obtained after this incubation time [46]. Some of the isolates were further subjected to fatty acid methyl ester (FAME) analysis. This analysis was performed by Five-Star Laboratories (Milford, CT).

2.6. Metabolic activity of the whole bacterial community

From each macerated root system (see above) a 1250-fold dilution in sterile distilled water was prepared. In order to reduce sample number, the suspensions from four root systems were pooled. Then BIOLOG GN microplates containing 95 different carbon sources (BIOLOG Inc., Hayward, CA) were inoculated with 100 μl per well of the pooled suspension. Microplates were shaken at 100 rpm at 24°C and the optical density at 570 nm of the cultures was measured after 35 h with an ELISA plate reader. Six microplates per treatment and repetition were used. The metabolic activity was assessed at both sampling dates at 10 and at 52 days.

2.7. Microcosms with a synthetic bacterial community

Based on the analysis of the rhizosphere community in natural soil, five bacterial isolates were selected which belonged to different dominant ARDRA groups and which varied in their sensitivity towards

the antibiotics Phl and Plt produced by CHA0-Rif. Sensitive isolates were selected from groups in which the majority of the isolates were sensitive, and resistant isolates were chosen from groups containing mainly resistant isolates (Table 3). The selected strains designated IM127, IM121, IM70, IM111 and IM116 are described in Tables 2 and 3. For inoculation into sterile soil the strains were grown overnight in TSB. Microcosms with a reconstituted synthetic bacterial community were set up as follows: 300 g of Eschikon soil was placed in 1-l Erlenmeyer flasks, autoclaved twice and inoculated with 20 ml of a suspension containing each 1.5×10^6 CFU ml^{-1} of IM127, IM121 and IM70 and each 1.5×10^7 CFU ml^{-1} of IM111 and IM116. The higher concentration of the latter two strains was used, since they colonized the soil more slowly. The soil was thoroughly mixed and the bacteria were allowed to colonize the soil for 5 days. Then 10 ml of a suspension with CHA0-Rif or CHA0-Rif/pME3424 was introduced into soil with a sterile syringe to obtain a final concentration of 10^7 CFU g^{-1} of soil. An equal volume of sterile distilled water was added to the control treatment. Two days later, the microcosms were planted with three 2-day-old surface-disinfested cucumber seedlings per flask. The incubation conditions were as described above for experiments in natural soil. After 2 weeks the plants were harvested, and culturable counts of the different bacterial strains were determined. Roots were macerated and the resulting homogenate was diluted and plated on the appropriate media as described above for the experiments in natural soil. All strains were distinguished from each other based on their colony morphology on TSA plates incubated at 27°C, with the exception of IM70 and CHA0-Rif, both having a similar colony morphology on TSA. Since IM70 is tryptophan side-chain oxidase (TSO)-negative, it could be distinguished from CHA0-Rif, which is TSO-positive, by using an agar overlay test for TSO activity [47]. In addition, CHA0-Rif and CHA0-Rif/pME3424 were enumerated on KMB containing 100 μg of rifampicin and 187.5 μg of actidione per ml and comparable results were obtained. Strains IM111 and IM116 were counted on additional TSA plates, which were incubated at 37°C; at this temperature only these two strains were able to form colonies. This procedure was chosen to prevent mistakes due to in vitro antibiosis on the dilution plates, since these two strains were unable to form enumerable colonies if CHA0-Rif or CHA0-Rif/3424 were present at high numbers on the same dilution plates.

2.8. Data analysis and statistics

Each experiment was repeated at different times. The whole experiment in microcosms with the natural bacterial community was repeated four times (sampling at 10 days) and three times (sampling at 52 days). Twenty-four plants were analyzed for each repetition and each sampling date. The experiment in microcosms with a synthetic bacterial community was repeated three times with six flasks per treatment and repetition, with the exception of the treatment with CHA0-Rif/pME3424, which was repeated twice. The data obtained for bacterial populations approximated a normal logarithmic distribution. Therefore, means, standard deviations and statistical tests of bacterial populations were calculated with $\log_{10}$-transformed values and data given in the text and in figures are back-transformed logarithmic means. Data from the different treatments were subjected to two-way analysis of variance (ANOVA; Systat 5.05 for windows, SPSS Inc., Evanston, IL), and if significant differences were found, the Tukey test was used for pairwise comparison of the treatments. BIOLOG data were linear-transformed by subtracting the average absorbance per plate from each value. This reduces variability due to color development in the control well (containing only the basal medium without a specific carbon source added), which occurred in several plates. A similar transformation was used by Garland and Mills [34]. Principal component analysis (PCA) on the BIOLOG data was performed based on the correlation matrix and using varimax rotation (Systat 5.05).

3. Results

3.1. Root colonization by introduced strains and total culturable bacteria

Root colonization by CHA0-Rif was 3.9×10^5 CFU g^{-1} of roots after 10 days, and then declined to about 4.5×10^4 CFU g^{-1} within the next 42 days.

Table 1
Impact of CHA0-Rif and CHA0-Rif/pME3424 on the metabolic activity of the whole bacterial community on glucose di- and polymers contained in BIOLOG GN plates

Treatment and sampling time[a]	α-Cyclodextrin	Dextrin	Maltose	Cellobiose	Glycogen	Gentiobiose
10 days						
No bacteria added	−0.081 a[b]	−0.082 a	−0.123 a	−0.279 a	−0.130 a	−0.030 a
CHA0-Rif	0.045 b***	0.044 b*	−0.036 b**	−0.181 b**	−0.061 a	0.058 a
CHA0-Rif/pME3424	0.066 b***	0.035 b*	−0.032 b**	−0.197 b**	−0.058 a	0.036 a
52 days						
No bacteria added	0.071 A	−0.011 A	−0.069 A	−0.095 A	−0.054 A	−0.038 A
CHA0-Rif	0.028 A	0.039 A	0.035 A	−0.010 A	−0.093 A	−0.003 A
CHA0-Rif/pME3424	0.022 A	−0.055 A	−0.107 A	−0.134 A	−0.104 A	0.069 A

Data are expressed as OD_{570} values after subtraction of the average absorbance per plate.
[a]CHA0-Rif and CHA0-Rif/pME3424 were introduced into natural soil at 10^7 CFU g^{-1} and 2-day-old cucumber seedlings were planted. Microcosms were incubated for 10 and 52 days. Roots were washed gently to remove loose soil and then macerated. Each BIOLOG GN plate was incubated with the pooled and diluted root macerate of four plants (100 μl per well). OD_{570} was measured after 35 h of incubation at 24°C. For details see Section 2.
[b]Values are means from four (10 days) and three (52 days) independent experiments with six microplates per treatment and experiment. Values were transformed by subtracting the average absorbance per plate from each value. Means with the same letter (letters a, b, and A) within the same column and sampling date are not significantly different at $P < 0.05$ (two-way ANOVA and Tukey test); significance levels are: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

The functionally improved derivative CHA0-Rif/pME3424 colonized the roots to a significantly lower extent ($P < 0.05$), and cell numbers were 2.5×10^5 and 2.3×10^4 CFU g^{-1}, respectively, at the two sampling dates. Plasmid pME3424 was maintained in 89% of the cells after 10 days and in 87% of the cells after 52 days (data not shown). The culturable bacterial population was essentially the same in all treatments at about 10^8 CFU g^{-1} at both sampling dates.

3.2. Impact on the metabolic activity of the whole bacterial community

The most obvious effect of CHA0-Rif and CHA0-Rif/pME3424 on the metabolic activity of the whole bacterial community was an increase in both inoculated treatments after 10 days on all six glucose di- and polymers included in BIOLOG GN microplates (α-cyclodextrin, dextrin, maltose, cellobiose, glycogen and gentiobiose) (Table 1). This effect was highly significant for α-cyclodextrin ($P < 0.001$), maltose and cellobiose ($P < 0.01$) and significant ($P < 0.05$) for dextrin (Table 1). Interestingly, the same effect was observed if either CHA0-Rif or CHA0-Rif/pME3424 was introduced. This effect of the two inoculants was not detectable when the metabolic activity of the bacterial community was evaluated 52 days after inoculation (Table 1). CHA0-Rif itself

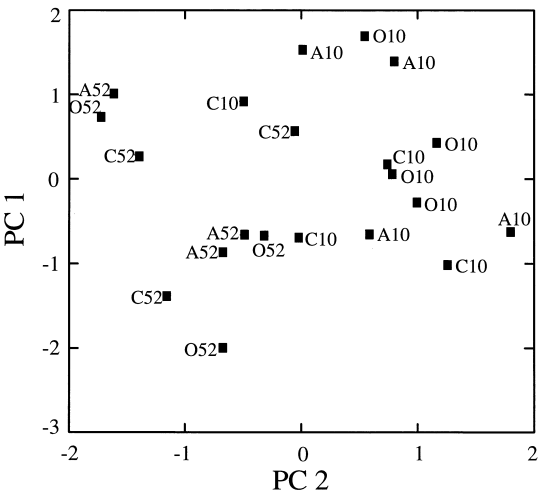

Fig. 1. Impact of CHA0-Rif, CHA0-Rif/pME3424 and plant age on the metabolic activity of the bacterial community as measured with BIOLOG GN plates containing 95 different carbon sources after 10 days and 52 days. Average values of each treatment and each repetition are plotted along the first two principal components (PC 1 and PC 2) resulting from principal component analysis. C10, A10, O10: control, CHA0-Rif and CHA0-Rif/pME3424, respectively, after 10 days; C52, A52, O52: control, CHA0-Rif and CHA0-Rif/pME3424, respectively, after 52 days. 33.5% and 12.5%, respectively, of the total variance are explained by the first two components. For details see Section 2.

Table 2
Characterization of selected bacterial strains representing dominant ARDRA groups of the resident bacterial community on cucumber roots

Strain designation	ARDRA group[a]	Percentage of isolates included in group	Size of restriction fragments with *Cfo*I	Gram reaction[b]	FAME identification[c]	BIOLOG identification[d]
IM121	B			−	n.g.	n.r.
IM27	B	11.4	550 500 360 70	−	n.g.	n.r.
IM140	B			−	n.g.	n.r.
IM54	C			−	*Variovorax paradoxus*	*Variovorax paradoxus*
IM55	C	5.7	550 410 380 140 70	−	*Yersinia pseudotuberculosis*	*Variovorax paradoxus*
IM52	C			−	*Variovorax paradoxus*	*Variovorax paradoxus*
IM126	D			−	n.g.	*Weeksella zoohelcum*
IM127	D	12.5	550 300 210 210 140 70	−	n.g.	*Sphingomonas paucimobilis B*
IM143	D			−	n.g.	*Capnocytophaga gingivalis*
IM36	E			−	*Acidovorax delafielda*	n.i.
IM35	E	4.3	550 410 380 200	−	*Acidovorax facilis*	*Acidovorax facilis A*
IM146	E			−	*Alcaligenes xylosoxydans*	*Pseudomonas vesicularis*
IM144	G			−	*Pseudomonas syringae*	n.r.
IM145	G	10.3	550 360 300 210 70	−	n.g.	n.r.
IM141	G			−	n.g.	n.r.
IM46	N			−	*Janthinobacterium lividum*	n.r.
IM142	N	5.2	550 360 360 210 70	−	n.g.	n.r.
IM147	N			−	n.g.	n.r.
IM134	P			−	*Cytophaga johnsonae*	n.i.
IM148	P	9.9	730 410 290 90	−	n.g.	*Pasteurella pneumotropica heyl*
IM149	P			−	*Cytophaga johnsonae*	*Pasteurella pneumotropica heyl*
IM70	R			−	*Pseudomonas chlororaphis*	*Pseudomonas fluorescens* type G
IM73	R	3.5	410 360 290 260 200	−	*Pseudomonas putida*	*Pseudomonas fluorescens* type G
IM71	R			−	*Pseudomonas putida*	*Pseudomonas fluorescens* type G
IM116	U			+	*Bacillus sphaericus*	n.i.
IM34	U	5.5	410 350 340 230 190	+	*Bacillus circulans*	n.r.
IM40	U			+	*Paenibacillus polymyxa*	n.i.
IM111	W			+	*Bacillus mycoides*	n.r.
IM115	W	11.9	580 410 340 190	+	*Paenibacillus polymyxa*	n.r.
IM18	W			+	*Bacillus cereus*	n.r.
IM62	K			−	*Agrobacterium rubi*	n.d.
IM63	K	4.6	330 330 290 180 140 120 70	−	*Agrobacterium radiobacter*	*Agrobacterium rhizogenes A*
IM68	K			−	*Agrobacterium radiobacter*	*Agrobacterium rhizogenes A*

[a]From each of the 11 dominant ARDRA groups three isolates were chosen for further characterization. The isolates used for the experiments with a synthetic bacterial community are highlighted with bold letters.
[b]Gram reaction was determined with the KOH string test [54].
[c]FAME analysis was performed by Five-Stars Laboratories (Milford, CT); n.g., no growth.
[d]BIOLOG identification was performed as indicated by the manufacturer, with the exception that plates were read after 72 h; n.d., not done; n.i., no identification at the 0.50 similarity level recommended by the manufacturer; n.r., all wells negative.

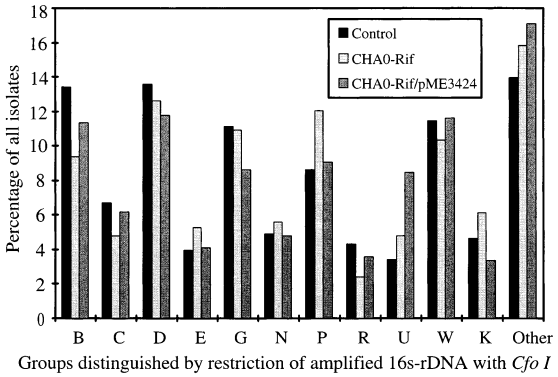

Fig. 2. Influence of CHA0-Rif and CHA0-Rif/pME3424 on the frequency of the 11 prevalent genotypic groups of bacteria on cucumber roots as distinguished by restriction analysis of amplified 16S rDNA with the restriction enzyme *Cfo*I. Natural soil was inoculated with 10^7 CFU g^{-1} of either CHA0-Rif or CHA0-Rif/pME3424 or left untreated (control), and then planted with cucumber. Roots were harvested, gently washed to remove loose soil and macerated after 10 days of growth and bacteria were isolated on TSA. Bars represent the means from four experiments with a total of 2500 isolates (about 200 isolates per repetition and per treatment) which were successfully stored, amplified and restricted. None of the differences between the treatments and within the same ARDRA group are significant at $P < 0.05$ (ANOVA and Tukey test).

cannot degrade these glucose di- and polymers (data not shown).

To compare the effect of plant age with the effect of inoculation, PCA was applied to all data after 10 and after 52 days and for all 95 substrates included in the BIOLOG GN plates. Fig. 1 shows the ordination plot of the first two components for the average values of each treatment and repetition; 33.5% and 12.5% of the variance could be explained by the first two components. The data from the different treatments were not separated by this procedure, indicating that the variance in the overall carbon source utilization on the 95 substrates of the BIOLOG GN plates between different repetitions is more pronounced than the overall effects due to the inoculant (Fig. 1). On the other hand, there was a clear separation of the data from the two sampling dates along the two principal component axes. This indicates that changes in the metabolic activity of the root-associated bacterial community during plant growth were more pronounced than the changes induced by inoculation (Fig. 1). This finding was confirmed by comparing the data for each carbon source individually: for 40 of the 95 carbon sources a significant difference between the two sampling dates was found. On the other hand, after 10 days a significant difference was found between inoculated and untreated roots for only 24 carbon sources.

3.3. *Effects on dominant genotypic groups of culturable resident bacteria discriminated with ARDRA*

Restriction of 16S rDNA of rifampicin-sensitive TSA isolates was used to investigate the population structure of the root-colonizing resident bacteria after 10 days of plant growth. Out of 3300 isolates, 76% could be successfully stored, amplified and restricted (i.e. about 210 isolates per treatment and repetition). Restriction analysis with *Cfo*I distinguished 11 dominant groups of root bacteria sharing all restriction fragments and accounting for 82% of the evaluated isolates (Table 2, Fig. 2). Sixteen further fingerprints accounted for another 11% of the isolates, and about 15 other distinct fingerprints were observed only occasionally. A second restriction with *Taq*I was performed on the first 1000 isolates, but only a few more dominant groups were discriminated (data not shown). Compared with the untreated control, inoculation with CHA0-Rif or CHA0-Rif/pME3424 had no significant effects on the frequency of the different ARDRA groups (Fig. 2), neither were differences observed between the two inoculants. The Shannon index of diversity was calculated based on the frequency of the different restriction groups, but no significant differences were detected between the treatments (data not shown). Since the density of CHA0-Rif declined between the two sampling dates, and no significant effects of the inoculants were observed after 10 days, the isolates obtained after 52 days were not subjected to ARDRA.

3.4. *Effects on culturable bacteria which are sensitive to the antibiotics Plt and Phl*

The minimal inhibitory concentration of Phl and Plt and the in vitro inhibition by CHA0-Rif and CHA0-Rif/pME3424 was assessed for three isolates of each ARDRA group. In general, the resistance to the antibiotics was similar for isolates from the same

Table 3
Sensitivity to Phl and Plt and in vitro inhibition by CHA0-Rif and CHA0-Rif/pME3424 of selected bacterial isolates from cucumber roots representing dominant ARDRA groups (see Table 2)

Strain designation	ARDRA group[a]	MIC Phl[b]	MIC Plt[b]	Inhibition by CHA0-Rif in vitro[c]	Inhibition by CHA0-Rif/pME3424 in vitro[c]	Percentage of isolates within the group sensitive to 5 μg ml^{-1} Phl	Percentage of isolates within the group sensitive to 5 μg ml^{-1} Plt
IM121	B	100	10	3.6 x	7.6 y		
IM27	B	50	5	3.3 x	6.6 y	14.3	35.4
IM140	B	50	5	5.2 x	8.0 y		
IM54	C	50	5	3.6 x	7.0 y		
IM55	C	100	10	< 2 x	< 2 x	3.8	28.8
IM52	C	50	5	< 2 x	< 2 x		
IM126	D	100	10	3.2 x	7.8 y		
IM127	D	100	25	3.0 x	5.2 y	13.5	19.6
IM143	D	100	10	< 2 x	< 2 x		
IM36	E	50	5	3.1 x	4.2 x		
IM35	E	50	5	n.d.	n.d.	26.5	51.9
IM146	E	100	25	< 2 x	< 2 x		
IM144	G	100	10	12.9 x	15.8 y		
IM145	G	100	5	2.6 x	3.4 x	33.5	57.2
IM141	G	100	5	3.0 x	4.4 x		
IM46	N	100	10	5.8 x	7.7 x		
IM142	N	100	10	< 2 x	< 2 x	16.2	39.0
IM147	N	200	25	2.7 x	3.7 y		
IM134	P	100	5	8.0 x	10.6 y		
IM148	P	100	5	10.2 x	11.9 y	57.2	61.9
IM149	P	100	5	10.1 x	12.4 y		
IM70	R	> 200	> 200	< 2 x	< 2 x		
IM73	R	> 200	200	< 2 x	< 2 x	3.1	11.1
IM71	R	> 200	> 200	< 2 x	< 2 x		
IM116	U	1	5	15.9 x	16.6 x		
IM34	U	n.g.	n.g.	18.6 x	23.4 y	94.1	85.2
IM40	U	100	1	8.1 x	11.6 y		
IM111	W	< 0.5	2	12.4 x	18.8 y		
IM115	W	< 0.5	2	14.2 x	21.3 y	82.0	70.7
IM18	W	< 0.5	5	13.1 x	18.2 y		
IM62	K	200	10	3.25 x	5.8 y		
IM63	K	200	25	< 2 x	< 2 x	10.7	17.9
IM68	K	200	25	< 2 x	< 2 x		

[a]From each of the 11 dominant ARDRA groups three isolates were chosen for further characterization. The isolates used in the experiments with the synthetic community are highlighted with bold letters.
[b]The minimal inhibitory concentrations (MIC) of Phl and Plt were assessed by growing the strains in 100 μl of TSB containing the following concentrations of the antibiotics: 0.25, 0.5, 1, 2, 5, 10, 25, 50, 100, 200 μg ml^{-1}. Growth was determined by measuring OD_{570} after 3 days with an ELISA plate reader. Values below 0.05 were considered no growth; n.g., no sufficient growth in wells without antibiotic.
[c]Each value is the mean from six replicates. Means with different letters in the same row are significantly different at $P < 0.05$ (ANOVA and Tukey test). n.d., not done, for details see Section 2.

group (Table 3). Most of the isolates which were strongly inhibited by CHA0-Rif in vitro were significantly more inhibited by CHA0-Rif/pME3424. To assess whether the abundance of antibiotic sensitive bacteria on the roots is affected by the inoculants, the sensitivity of all isolates to Phl and Plt was measured. About 30% of the isolates from the resident bacterial root population could not grow in the presence of 1 μg ml^{-1} of Phl (Table 4) and about 55% of the isolates were inhibited by 5 μg ml^{-1} of Plt. A

Table 4
Impact of CHA0-Rif and CHA0-Rif/pME3424 on the resistance of resident bacteria to the antibiotics Phl and Plt

Treatment[a]	Log of total CFU g^{-1}	Percentage of isolates resistant against[b]				
		Phl 1 μg ml^{-1}	Phl 5 μg ml^{-1}	Phl 50 μg ml^{-1}	Plt 5 μg ml^{-1}	Plt 50 μg ml^{-1}
No bacteria added	8.03 ± 0.08	68.4 ± 15.0	65.9 ± 10.3	42.5 ± 9.9	51.1 ± 4.9	6.6 ± 1.2
CHA0-Rif	8.07 ± 0.10	69.6 ± 17.2	69.7 ± 18.8	44.1 ± 18.0	51.8 ± 8.5	5.7 ± 4.4
CHA0-Rif/pME3424	8.02 ± 0.11	69.8 ± 11.8	69.8 ± 12.1	50.1 ± 9.3	59.5 ± 9.7	8.6 ± 4.3

Resistance of individual TSA isolates obtained from the roots of 10-day-old cucumber plants was assessed.
[a]For details of the experimental setup see legend to Table 1 and Section 2.
[b]Values are means (± standard deviation) from four experiments with 24 plants per treatment and repetition, with a total of 3300 isolates (12 isolates per root system). Resistance tests were performed in microtiter plates with 100 μl TSB per well containing the appropriate amount of Phl or Plt and read after 3 days with an ELISA reader. $OD_{570} \geq 0.05$ was scored as positive growth. The differences between the treatments within the same column were not statistically significant at $P < 0.05$ according to ANOVA and the Tukey test.

large proportion of the sensitive isolates belonged to the same two ARDRA groups U and W (Table 3). Inoculation with either CHA0-Rif or CHA0-Rif/pME3424 did not affect the sensitive subpopulation in situ and sensitive isolates were recovered at the same frequency from inoculated and control plants (Table 4). The isolates obtained after 52 days were therefore not scored for antibiotic resistance.

3.5. *Identification of selected isolates from the different ARDRA groups*

Three independent isolates from each of the 11 dominant groups distinguished with ARDRA were chosen for identification with BIOLOG and FAME (Table 2). The BIOLOG system could not identify all isolates. Only 18 of the 33 isolates gave positive reactions and matched species of the BIOLOG database and some of the isolates were able to grow on the medium used for FAME analysis (Table 2). However, within most ARDRA groups the same or a similar genus was obtained for the investigated isolates, indicating that ARDRA was a useful approach for rapid determination of dominant taxonomic groups (Table 2).

3.6. *Impact on different strains in a synthetic bacterial community*

To determine whether CHA0-Rif and CHA0-Rif/pME3424 affect the highly sensitive bacteria under more simplified and controlled conditions, microcosms containing a synthetic population consisting of five bacterial strains belonging to different dominant ARDRA groups were established. The five strains colonized cucumber roots effectively in the microcosms, although the population densities of the sensitive bacteria IM111 and IM116 reached only about 20% of those of the other strains (Table 5). The number of cells of CHA0-Rif and CHA0-Rif/pME3424 was 7.1×10^7 and 8.3×10^7 CFU g^{-1} roots, respectively (Table 5, Fig. 3), and thus more than 100-fold higher compared to the microcosms with the natural resident community. Culturable counts of strain IM70, which was identified as a strain of *Pseudomonas* (Table 2), and which is resist-

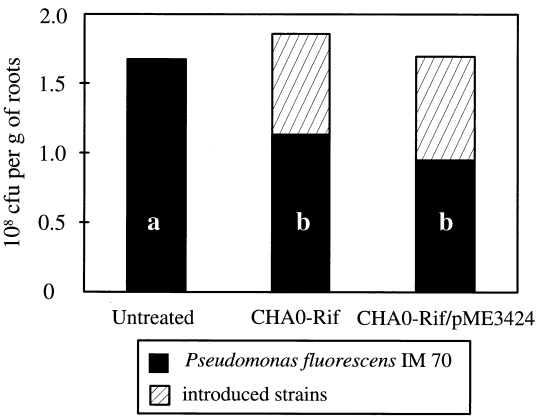

Fig. 3. Effect of CHA0-Rif and CHA0-Rif/pME3424 on the colonization of cucumber roots by *P. fluorescens* IM70 in microcosms with a synthetic bacterial community consisting of five bacterial strains after 2 weeks. Values are means from three (untreated and CHA0-Rif) and two (CHA0-Rif/pME3424) independent experiments with six microcosms per treatment and experiment. Filled bars with different letters are significantly different at $P < 0.05$ as indicated by two-way ANOVA and the Tukey test. For details see Section 2.

Table 5
Influence of CHA0-Rif and CHA0-Rif/pME3424 on individual strains of a synthetic bacterial community associated with roots of cucumber plants grown for 2 weeks in microcosms[a]

Treatment	Introduced strain	Log CFU per g of roots of the different strains of a synthetic bacterial community				
		IM70	IM111	IM116	IM121	IM127
Untreated	0	8.22 ± 0.20 x	7.24 ± 0.12 x	7.31 ± 0.12 x	8.37 ± 0.14 x	7.92 ± 0.17 x
CHA0-Rif	7.85 ± 0.22	8.03 ± 0.24 y	7.16 ± 0.13 x	7.23 ± 0.16 x	8.31 ± 0.13 x	7.81 ± 0.17 x
CHA0-Rif/pME3424	7.92 ± 0.17	7.99 ± 0.23 y	7.25 ± 0.11 x	7.29 ± 0.14 x	8.31 ± 0.09 x	8.02 ± 0.20 x

[a]Five isolates from natural soil representing different dominant ARDRA groups were selected. The isolates are described in Tables 2 and 3. Autoclaved Eschikon soil (300 g) was inoculated with each 10^5 CFU g^{-1} of IM127, IM121, IM70 and with each 1×10^6 CFU g^{-1} of IM111 and IM116. The bacteria were allowed to colonize the soil for 5 days, and then CHA0-Rif or CHA0-Rif/pME3424 were introduced at 10^7 CFU g^{-1} of soil. Two days later, three cucumber seedlings per flask were planted. After 3 weeks of growth, the plants were harvested, and the different strains were enumerated as described in detail in Section 2. Values are means (± standard deviation) from three (untreated and CHA0-Rif) and two (CHA0-Rif/pME3424) independent experiments with six microcosms per treatment and experiment. Values with different letters in the same column are significantly different at $P < 0.05$ according to two-way ANOVA and the Tukey test.

ant to Phl and Plt (Table 3), were reduced significantly in both inoculated treatments. The reduction of the population was about 5.9×10^7 and 7.0×10^7 CFU g^{-1} of roots, respectively, in the presence of CHA0-Rif and CHA0-Rif/pME3424 (Table 5, Fig. 3). This corresponded nearly to the root colonization level attained by the introduced strains (Fig. 3), suggesting that CHA0-Rif or the mutant occupied part of the ecological niche colonized by IM70. In contrast, the antibiotic sensitive bacteria IM111 and IM116 (Table 3) reached the same population density in inoculated and uninoculated treatments (Table 5), although they were strongly inhibited by the inoculants CHA0-Rif and CHA0-Rif/pME3424 in vitro (Table 3). Both strains IM121 and IM127 with higher resistance to the antibiotics were not affected (Table 5). In conclusion, this finding confirmed the data from natural microcosms, showing that CHA0-Rif and its derivative CHA0-Rif/pME3424 did not affect the population size of antibiotic-sensitive bacteria.

4. Discussion

This study reports data about possible effects on the culturable root-colonizing bacterial community caused by the introduction into the soil ecosystem of a *Pseudomonas* biocontrol strain or its genetically engineered derivative with improved biocontrol activity. If significant effects appeared, they were compared to changes during plant growth.

The culturable bacterial counts on the roots were at about 10^8 CFU g^{-1}. This is very similar to the values found by both direct and plate counts by Foster et al. [27] and Miller et al. [28], and thus the culture-based approach to evaluate the community structure on plant roots seems to be justified.

Specific effects caused by CHA0-Rif and CHA0-Rif/pME3424 on the resident bacterial community were detected by evaluating the BIOLOG data for 95 different carbon sources. A significantly enhanced metabolic activity of the whole bacterial community on glucose di- and polymers molecules was observed in both inoculated treatments after 10 days. It should be noted that measuring the activity on 95 substrates will always lead to some significant results which occur by chance if each single carbon source is evaluated at the $P < 0.05$ significance level. However, several facts indicate that the observed effect was not due to random variation: (i) the effect was highly significant for three carbon sources, (ii) the same effect was observed for both inoculant strains and (iii) for all glucose di- and polymers included in BIOLOG plates. It seems that the effect was not due to an in vitro activity of the inoculant, since CHA0-Rif cannot grow on any of these substrates and a pure culture of CHA0-Rif does not induce a color reaction on these substrates in BIOLOG plates (data not shown). Furthermore, co-inoculation of BIOLOG plates with root macerate from uninoculated cucumber roots and a diluted culture of CHA0-Rif to obtain a ratio of CFU of resident bacteria:CFU of CHA0-Rif corresponding to that observed on inoculated roots after 10 days of growth did not provoke a

similar effect (unpublished results). We conclude therefore that the effect was due to an interaction in the rhizosphere rather than to in vitro interactions in the BIOLOG plates. Since CHA0-Rif cannot utilize these substrates, an enhanced selective pressure for the resident community to utilize these substrates which are not metabolized by the inoculant is a possible explanation for the observed effect. The BIOLOG assay has been used previously to study effects of a microbial inoculant [1,2]. Ellis et al. [1] found differences between bacterial communities grown in the presence or absence of a *Pseudomonas* inoculant in the phyllosphere of young sugar beet leaves. Gilbert et al. [29] assessed 45 physiological attributes of bacterial isolates with different media, and they found significant differences in the population structure on non-inoculated and *Bacillus cereus*-inoculated soybean roots.

Pronounced differences in the metabolic activity were found between the bacterial community present on cucumber roots after 10 and after 52 days. This could best be shown with PCA. The BIOLOG method in combination with PCA was applied by Garland and Mills [34] to discriminate between bacterial populations from various habitats (soil, water, hydroponic culture) and Insam et al. [48] to discriminate between the bacterial communities from different zones of compost windrows. Wünsche et al. [35] used this method to demonstrate effects of hydrocarbon pollution on the microbial community. Ellis et al. [1] found that the BIOLOG assay is able to discriminate between the bacterial community present on young and senescent leaves of sugar beet. The results of the present work show that, in addition, this method was sensitive enough to detect changes in the bacterial community on plant roots which seem to be due to natural succession within 6 weeks. These changes in the metabolic activity of the root-associated bacterial community during plant growth were more pronounced than the effects caused by the inoculants. This was evident from the fact that simultaneous evaluation of all carbon sources with PCA separated the data from the two sampling dates but not those from the different treatments along the first two principal component axes. A similar result had also been observed in a previous study on the effects of the inoculants on the pattern of carbon source utilization by resident pseudomonads: PCA also separated the two sampling dates, but not the different treatments, indicating that succession changes bacterial communities more than inoculation with CHA0-Rif and CHA0-Rif/pME3424 [42].

ARDRA analysis of individual bacterial isolates indicated that the proportion of dominant genotypic groups of culturable resident root bacteria was not significantly affected by inoculation with CHA0-Rif or CHA0-Rif/pME3424. At first sight, this contradicts somewhat the results from the metabolic profiles. Although the 1/10 strength TSA agar used in this study is usually the best choice as an isolation medium in an ecological study, it might still be selective and prevent the growth of certain bacterial species, whose abundance was affected by the inoculant strains. On the other hand, the resolution of the ARDRA technique with one restriction enzyme is moderate, and effects on the proportion of some subgroups of the dominant ARDRA groups cannot be excluded. Nevertheless, the resolution of this typing technique seems to be appropriate to determine dominant genotypic groups within a bacterial community, since with about 200 isolates per repetition and per treatment on average 10–20 isolates belong to each of the dominant groups, which still allows an adequate statistical analysis. Representative isolates of different ARDRA groups were identified with BIOLOG and FAME, and the results showed that the ARDRA method was useful for differentiation of dominant taxa. However, only part of the BIOLOG data corresponded at genus level with the FAME data, indicating that both methods identify only a part of environmental isolates, and a genotypic method such as ARDRA might be more reliable for rapid grouping of bacterial isolates in an ecological study. Regarding the most abundant taxa present in the soil used in this study and colonizing cucumber roots, results were similar to those reported recently by Wünsche and Babel [49], who found that the largest fraction of isolates identifiable with BIOLOG from an arable soil belonged to *Agrobacterium, Cytophaga, Pseudomonas, Xanthomonas* and *Bacillus*. Lilley et al. [32] investigated the bacterial population on sugar beet roots with FAME. Similar to our results, they found 10 dominant genera accounting for 70% of the isolates, and 30 other genera which were far less abundant.

Our finding that about 30–50% of the culturable

resident bacteria, in particular Gram-positive organisms, were sensitive to the antibiotics Phl and Plt produced by CHA0-Rif suggested that inoculant effects on these sensitive bacteria were likely, and that recombinant-specific effects of the antibiotic-overproducing derivative CHA0-Rif/pME3424 may occur. Nevertheless, neither of the inoculants reduced the proportion of these sensitive bacteria on the plant roots (Table 5). Our data are consistent with the work of Fukui et al. [50], who investigated interactions between pseudomonads in the spermosphere of sugar beets. In co-inoculation experiments with a strain producing an antibiotic metabolite and an antibiotic-sensitive strain, they found no inhibition of the sensitive strain in situ, although significant antibiosis occurred in vitro. On the other hand, our results contrast somewhat with the work of Kloepper and Schroth [51] who reported a reduced root population of Gram-positive bacteria 2 weeks after the introduction of a biocontrol strain producing an unidentified antibiotic as a seed treatment; they did not observe this effect if an antibiosis-negative mutant was applied.

The role of antibiotics produced by CHA0-Rif or other biocontrol bacteria in disease suppression is clearly documented. Production of antibiotics has been shown to be a key mechanism in the interaction between certain biocontrol strains and fungal pathogens [9,10]. In this context it is interesting that antibiotic-sensitive bacteria of ARDRA groups U and W were inhibited in vitro at antibiotic concentrations 20–100-fold lower than those inhibiting fungal pathogens [12] but their population was not affected in vivo. Since the amount of detectable antibiotics produced by biocontrol strains in the rhizosphere is extremely low [12,14–16], it has been postulated that antibiosis between biocontrol bacteria and pathogens occurs at specific microsites on the roots such as lesions generated by the pathogen, where nutrients are released [10,17]. It is possible that these niches are not colonized by bacteria which are sensitive to the antibiotics. Finally, spore formation immediately after an active growth phase could be a strategy of many Gram-positive bacteria to circumvent the deleterious effects of the antibiotics.

For the biological safety of biocontrol agents, the lack of interaction with *Bacillus* is an important issue since many *Bacillus* strains contribute positively to plant health and growth [52], and therefore negative effects on these species should not be neglected.

The lack of impact on antibiotic-sensitive organisms could be due to the relatively low root colonization level (4×10^5 CFU g^{-1}) attained by introduced CHA0-Rif and CHA0-Rif/pME3424. Therefore, additional experiments were conducted using microcosms with a synthetic bacterial community. In this system, both inoculants reached population densities that were over 100-fold higher than those observed in the microcosms with the natural community. Nevertheless, corresponding results were obtained, showing that an even higher root colonization by CHA0-Rif or its derivative did not affect most of the representative isolates of different ARDRA groups. Not even highly antibiotic sensitive isolates (IM111 and IM116), which are strongly inhibited by the inoculants in vitro, were affected in these microcosms. Interestingly, an exception was strain *P. fluorescens* IM70, which is highly resistant to the antibiotics in vitro and the population of which was significantly reduced in the presence of the inoculants. This effect may be attributed to competition for a similar ecological niche, since the reduction of the population of IM70 was similar to the population size of the introduced strains, and the total population of *Pseudomonas* (i.e. the sum of IM70 and the respective inoculant) did not differ significantly among the treatments (Fig. 3). A similar result has been found in a previous study on the effects of inoculation on the population of resident pseudomonads in natural soil [42]. In this context, De Leij et al. [53] suggested that fast-growing organisms such as fluorescent pseudomonads, which do not produce resting structures, are most sensitive to perturbations.

In conclusion, CHA0-Rif and its functionally improved derivative caused detectable perturbations in the metabolic activity of the resident bacterial community which were transient, similar for the wild-type and the GEM and less pronounced than changes due to natural succession. No evidence for deleterious effects of the biocontrol agent CHA0-Rif or its derivative on the fraction of culturable resident bacteria which are sensitive to Phl and Plt was found, and dominant genotypic groups of culturable bacteria were not affected by the inoculant. From a biological safety assessment point of view, our data

therefore suggest that a genetically improved biocontrol strain may have specific interactions with fungal pathogens rather than general effects on bacterial communities. However, we cannot dismiss the possibility that specific interactions could occur between introduced strains and non-culturable resident bacteria.

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References

[1] Ellis, R.J., Thompson, I.P. and Bailey, M.J. (1995) Metabolic profiling as a means of characterizing plant-associated microbial communities. FEMS Microbiol. Ecol. 16, 9–18.

[2] England, L.S., Hung, L. and Trevors, J.T. (1995) Recombinant and wild-type *Pseudomonas aureofaciens* strains introduced into soil microcosms: effect on decomposition of cellulose and straw. Mol. Ecol. 4, 221–230.

[3] Jones, R.A., Broder, M.W. and Stotzky, G. (1991) Effects of genetically engineered microorganisms on nitrogen transformations and nitrogen-transforming microbial populations in soil. Appl. Environ. Microbiol. 57, 3212–3219.

[4] Orvos, D.R., Lacy, G.H. and Cairns, J., Jr. (1990) Genetically engineered *Erwinia carotovora*: survival, intraspecific competition, and effects upon selected bacterial genera. Appl. Environ. Microbiol. 56, 1689–1694.

[5] Scanferlato, V.S., Orvos, D.R., Cairns, J., Jr. and Lacy, G.H. (1989) Genetically engineered *Erwinia carotovora* in aquatic microcosms: survival and effects on functional groups of indigenous bacteria. Appl. Environ. Microbiol. 55, 1477–1482.

[6] Crawford, D.L., Doyle, J.D., Wang, Z., Hendricks, C.W., Bentjen, S.A., Bolton, H., Jr., Fredrickson, J.K. and Bleakley, B.H. (1993) Effects of a lignin peroxidase-expressing recombinant, *Streptomyces lividans* TK23.1, on biogeochemical cycling and the numbers and activities of microorganisms in soil. Appl. Environ. Microbiol. 59, 508–518.

[7] Doyle, J.D., Short, K.A., Stotzky, G., King, R.J., Seidler, R.J. and Olsen, R.H. (1991) Ecologically significant effects of *Pseudomonas putida* PPO301(pRO103), genetically engineered to degrade 2,4-dichlorophenoxyacetate, on microbial populations and processes in soil. Can. J. Microbiol. 37, 682–691.

[8] Short, K.A., Doyle, J.D., King, R.J., Seidler, R.J., Stotzky, G. and Olsen, R.H. (1991) Effects of 2,4-dichlorophenol, a metabolite of a genetically engineered bacterium, and 2,4-dichlorophenoxyacetate on some microorganism-mediated ecological processes in soil. Appl. Environ. Microbiol. 57, 412–418.

[9] Cook, R.J., Thomashow, L.S., Weller, D.M., Fujimoto, D., Mazzola, M., Bangera, G. and Kim, D. (1995) Molecular mechanisms of defense by rhizobacteria against root diseases. Proc. Natl. Acad. Sci. USA 92, 4197–4201.

[10] Défago, G. and Keel, C. (1995) Pseudomonads as biocontrol agents of diseases caused by soil-borne pathogens. In: Benefits and Risks of Introducing Biocontrol Agents (Hokkanen, H.M.T. and Lynch, J.M., Eds.), pp. 137–148. Cambridge University Press, Cambridge.

[11] Keel, C. and Défago, G. (1997) Interactions between beneficial soil bacteria and root pathogens: Mechanisms and ecological impact. In: Multitrophic Interactions in Terrestrial Systems (Gange, A.C. and Brown, V.K., Eds.), pp. 27–46. Blackwell Science, London.

[12] Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. and Défago, G. (1992) Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol. Plant-Microbe Interact. 5, 4–13.

[13] Nowak-Thompson, B., Gould, S.J., Kraus, J. and Loper, J.E. (1994) Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can. J. Microbiol. 40, 1064–1066.

[14] Kempf, H.J., Sinterhauf, S., Müller, M. and Pachlatko, P. (1994) Production of two antibiotics by a biocontrol bacterium in the spermosphere of barley and in the rhizosphere of cotton. In: Improving Plant Productivity with Rhizosphere Bacteria (Ryder, M.H., Stephens, P.M. and Bowen, G.D., Eds.), pp. 114–116. CSIRO Division of Soils, Adelaide.

[15] Maurhofer, M., Keel, C., Haas D. and Défago, G. (1995) Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHA0 with enhanced antibiotic production. Plant Pathol. 44, 40–50.

[16] Thomashow, L.S., Weller, D.M., Bonsall, R.F. and Pierson III, L.S. (1990) Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol. 56, 908–912.

[17] Weller, D.M. and Thomashow, L.S. (1990) Antibiotics: evidence for their production and sites where they are produced. In: New Directions in Biological Control (Baker, R.R. and Dunn, P.E., Eds.), pp. 703–711. Alan R. Liss, New York.

[18] Bangera, M.G. and Thomashow, L.S. (1996) Characterization of a genomic locus required for synthesis of the antibiotic 2,4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. Mol. Plant-Microbe Interact. 9, 83–90.

[19] Fenton, A.M., Stephens, P.M., Crowley, J., O'Callaghan, M. and O'Gara, F. (1992) Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl. Environ. Microbiol. 58, 3873–3878.

[20] Hill, D.S., Stein, J.I., Torkewitz, N.R., Morse, A.M., Howell, C.R., Pachlatko, J.P., Becker, J.O. and Ligon, J.M. (1994) Cloning of genes involved in the synthesis of pyrrolnitrin from *Pseudomonas fluorescens* and role of pyrrolnitrin synthesis in biological control of plant disease. Appl. Environ. Microbiol. 60, 78–85.

[21] Pierson, L.S., III and Thomashow, L.S. (1992) Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Mol. Plant-Microbe Interact. 5, 330–339.

[22] Sarniguet, A., Kraus, J., Henkels, M.D., Muehlchen, A.M. and Loper, J.E. (1995) The sigma factor σ^s affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. Proc. Natl. Acad. Sci. USA 92, 12255–12259.

[23] Schnider, U., Keel, C., Blumer, C., Troxler, J., Défago, G. and Haas, D. (1995) Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J. Bacteriol. 177, 5387–5392.

[24] Voisard, C., Keel, C., Haas, D. and Défago, G. (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. 8, 351–358.

[25] Akkermans, A.D.L., Van Elsas, J.D. and De Bruijn, F.J. (Eds.) (1995) Molecular Microbial Ecology Manual. Kluwer Academic, Dordrecht.

[26] Frederickson, J.K., Bolton, H. and Stotzky, G. (1992) Methods for evaluating the effects of microorganisms on biogeochemical cycling. In: Microbial Ecology: Principles, Methods and Applications (Levin, M.A., Seidler, R.J. and Rogul, M., Eds.), pp. 579–606. McGraw-Hill, New York.

[27] Foster, R.C., Rovira, A.D. and Cook, T.W. (1983) Ultrastructure of the Root-Soil Interface. American Phytopathology Society, St. Paul, MN.

[28] Miller, H.J., Henken, G. and van Veen, J.A. (1989) Variation and composition of bacterial populations in the rhizospheres of maize, wheat, and grass cultivars. Can. J. Microbiol. 35, 656–660.

[29] Gilbert, G.S., Parke, J.L., Clayton, M.K. and Handelsman, J. (1993) Effects of an introduced bacterium on bacterial communities on roots. Ecology 74, 840–854.

[30] Laguerre, G., Rigottier, G.L. and Lemanceau, P. (1994) Fluorescent *Pseudomonas* species categorized by using polymerase chain reaction (PCR)/restriction fragment analysis of 16S rDNA. Mol. Ecol. 3, 479–487.

[31] Latour, X., Corberand, T., Laguerre, G., Allard, F. and Lemanceau, P. (1996) The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. Appl. Environ. Microbiol. 62, 2449–2456.

[32] Lilley, A.K., Fry, J.C., Bailey, M.J. and Day, M.J. (1996) Comparison of aerobic heterotrophic taxa isolated from four root domains of mature sugar beet (*Beta vulgaris*). FEMS Microbiol. Ecol. 21, 231–242.

[33] Paffetti, D., Scotti, C., Gnocchi, S., Fancelli, S. and Bazzicalupo, M. (1996) Genetic diversity of an italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. Appl. Environ. Microbiol. 62, 2279–2285.

[34] Garland, J.L. and Mills, A.L. (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol. 57, 2351–2359.

[35] Wünsche, L., Brüggemann, L. and Babel, W. (1995) Determination of substrate utilization patterns of soil microbial communities: An approach to assess population changes after hydrocarbon pollution. FEMS Microbiol. Ecol. 17, 295–305.

[36] Maurhofer, M., Keel, C., Schnider, U., Voisard, C., Haas, D. and Défago, G. (1992) Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology 82, 190–195.

[37] Natsch, A., Keel, C., Pfirter, H.A., Haas, D. and Défago, G. (1994) Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHA0 introduced into soil microcosms. Appl. Environ. Microbiol. 60, 2553–2560.

[38] Natsch, A., Keel, C., Troxler, J., Zala, M., von Albertini, N. and Défago, G. (1996) Importance of preferential flow and soil management in vertical transport of a biocontrol strain of *Pseudomonas fluorescens* in structured field soil. Appl. Environ. Microbiol. 62, 33–40.

[39] Troxler, J., Zala, M., Natsch, A., Moënne-Loccoz, Y. and Défago, G. (1997) Autecology of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the rhizosphere and inside roots at later stages of plant development. FEMS Microbiol. Ecol. 23, 119–130.

[40] Troxler, J., Zala, M., Moënne-Loccoz, Y., Keel, C. and Défago, G. (1997) Long-term persistence of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the surface horizon of large outdoor lysimeters. Appl. Environ. Microbiol. 63, 3776–3782.

[41] Troxler, J., Azelvandre, P., Zala, M., Défago, G. and Haas, D. (1997) Conjugative transfer of chromosomal genes between fluorescent pseudomonads in the rhizosphere of wheat. Appl. Environ. Microbiol. 63, 213–219.

[42] Natsch, A., Keel, C., Hebecker, N., Laasik, E. and Défago, G. (1997) Influence of biocontrol strain *Pseudomonas fluorescens* CHA0 and its antibiotic overproducing derivative on the diversity of resident root colonizing pseudomonads. FEMS Microbiol. Ecol. 23, 341–352.

[43] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

[44] King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44, 301–307.

[45] Keel, C., Weller, D.M., Natsch, A., Défago, G., Cook, R.J. and Thomashow, L.S. (1996) Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudo-*

monas strains from diverse geographic locations. Appl. Environ. Microbiol. 62, 552–563.

[46] Tonso, N.L., Matheson, V.G. and Holben, W.E. (1995) Polyphasic characterization of a suite of bacterial isolates capable of degrading 2,4-D. Microb. Ecol. 30, 3–24.

[47] Takai, K. and Hayaishi, O. (1987) Purification and properties of tryptophan side chain oxidase types I and II from *Pseudomonas*. Methods Enzymol. 142, 195–217.

[48] Insam, H., Amor, K., Renner, M. and Crepaz, C. (1996) Changes in functional abilities of the microbial community during composting of manure. Microb. Ecol. 31, 77–87.

[49] Wünsche, L. and Babel, W. (1996) The suitability of the Biolog automated microbial identification system for assessing the taxonomical composition of terrestrial bacterial communities. Microbiol. Res. 151, 133–143.

[50] Fukui, R., Schroth, M.N., Hendson, M. and Hancock, J.G. (1994) Interactions between strains of pseudomonads in sugar beet spermatospheres and their relationship to pericarp colonization by *Pythium ultimum* in soil. Phytopathology 84, 1322–1330.

[51] Kloepper, J.W. and Schroth, M.N. (1981) Relationship of in vitro antibiosis of plant growth-promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology 71, 1020–1024.

[52] Stabb, E.V., Jacobson, L.M. and Handelsman, J. (1994) Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. Appl. Environ. Microbiol. 60, 4404–4412.

[53] De Leij, F.A.A.M., Sutton, E.J., Whipps, J.M., Fenlon, J.S. and Lynch, J.M. (1995) Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. Appl. Environ. Microbiol. 61, 3443–3453.

[54] Powers, E.M. (1995) Efficacy of the Ryu nonstaining KOH technique for rapidly determining Gram reactions of food-borne and waterborne bacteria and yeasts. Appl. Environ. Microbiol. 61, 3756–3758.